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Maintaining the artemisinin content through direct and indirect *in vitro* regeneration and their assessment of variations with the field grown mother plants of *Artemisia annua* L.

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Abstract: An efficient protocol for maintaining the artemisinin content in tissue culture and high frequency of *in vitro* direct and indirect regenerations of multiple shoots of high artemisinin yielding genotypes of *Artemisia annua* has been developed and their comparison with field grown mother plant has been carried out. The eleven elite genotypes (containing more than 1% artemisinin) were tested on seven different modified media formulations. Modified half MS (Murashige and Skoog's) media containing 100 mg L^{-1} myo-inositol, 0.5 g L^{-1} casine hydrolysate, 5 mg L^{-1} biotin, 2 mL L^{-1} RT (Revised Tobacco) vitamin stock, 0.5 mg L^{-1} BAP and 0.01 mg L^{-1} NAA showed best regeneration while, above modified MS medium containing 0.2 mg L^{-1} BAP and 0.2 mg L^{-1} NAA showed maximum shoot multiplication with maintained artemisinin content. Based on the chemical profiles of both the systems, minor difference was observed in their artemisinin content. A large scale culture of these plants maintained the normal growth index along with the artemisinin content and could be a better alternative to maintain the high artemisinin yielding genotypes with their genetic constraint in specific media combinations and also used as base material for further genetic improvement.

Keywords: direct and indirect regeneration, *Artemisia annua*, artemisinin, BAP, NAA

Introduction

Malaria still remains a deadly disease in most of the developing nations which claims about 1–2 million deaths every year. World Health Organization currently recommends the artemisinin-based combination therapies (ACTs) in tropical regions where the malarial parasite seems to have developed resistance to traditional anti-malarial drugs.[1] Drugs that contain artemisinin derivatives have almost 100% cure rates with usually only a three-day regimen.[2] All stages of the malarial parasite are killed by artemisinin derivatives by interacting with heme in the blood and damaging the parasitic cells, micro-organelles and membranes[3]. The artemisinin-derived molecules are artesunate, arteether, artemether, and dihydro-artemisinin (DHA).[4] These compounds have a particularly rapid clearance time (the rate at which a substance is removed from the blood) and making them suitable for treating early cases of malaria which is used to avoid progressions toward severe cases that lead to mortality in developing countries[1]. Therefore, increasing efficiency in artemisinin production by different ways like breeding, cell and *in vitro* culture to reduce costs of artemisinin-based medications is an important step in addressing the global spread of malaria.[5]

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Artemisinin is a sesqueterpene lactone found in the trichomes of *Artemisia annua*. The species *Artemisia annua* L. (Asteraceae, 2n = 18) is native to China. In natural sources the concentration of artemisinin various from 0.1 to 1.2%. The highest artemisinin content has been found in leaves and flowering tops of the plant and it is influenced by several factors such as plant variety, cultivation conditions and geographic localization.[6,7,8] Various workers have carried out experiments in order to investigate production of artemisinin by alternate means. Martinez and Staba[9] and Elhag et al.[10] investigated the production of artemisinin through tissue culture of *A. annua.* A comparative evaluation of the artemisinin content was also studied in tissue culture, green house and field grown plants of *Artemisia* by Ferreira et al.[7] Artemisinin was observed to be produced by differentiated shoot culture[11,12,13] but only at trace level.[9,14,15,16]

Selection for high-producing lines through traditional breeding, and research on the effects of environmental conditions and cultural practices could perhaps lead to an improvement of artemisinin content,[7,17,18,19] as its maintenance in the natural population is difficult due to its open pollinated nature. Therefore, it exhibits a great range of variability in its artemisinin yield in field condition due to various environmental and agricultural factors. *In vitro* cultures of high yielding genotypes can be a promising alternative to maintain high producing lines, which can be used to multiply in tissue culture and reintroduce in field for artemisinin production. Therefore, an attempt has been made to i) establish

high yielding genotypes in tissue culture (direct and indirect regeneration) ii) study the effects of plant growth regulators on growth, tissue differentiation and artemisinin production iii) compare the chemical and molecular diversity between *in vitro* cultured and field grown mother plants.

Results and Discussion

Two different explants systems i.e., nodal and leaves segments from eleven selected high yielding (more than 1% artemisinin) genotypes were cultured on the modified ½MS medium supplemented with different hormonal combinations of BAP and NAA, in order to select the best supportive media for direct and indirect regenerations. Media containing 0.2 mg L^{-1} BAP and 0.05 mg L^{-1} NAA showed 84% regeneration from nodal segments, 95% regeneration frequency from leaf explants was found in 0.5 mg L^{-1} BAP and 0.01 mg L^{-1} NAA. 84% nodal proliferation was observed in 0.2 mg L^{-1} BAP and 0.2 mg L^{-1} NAA (Table 2). Among the BAP and NAA combinations tried in the present investigation, the nodal explants, when cultured on the MS medium containing 0.1 mg L^{-1} BAP and 0.05 mg L^{-1} NAA, showed pale green friable callus at the margins of the explants (Table 1 and Figure 1a).

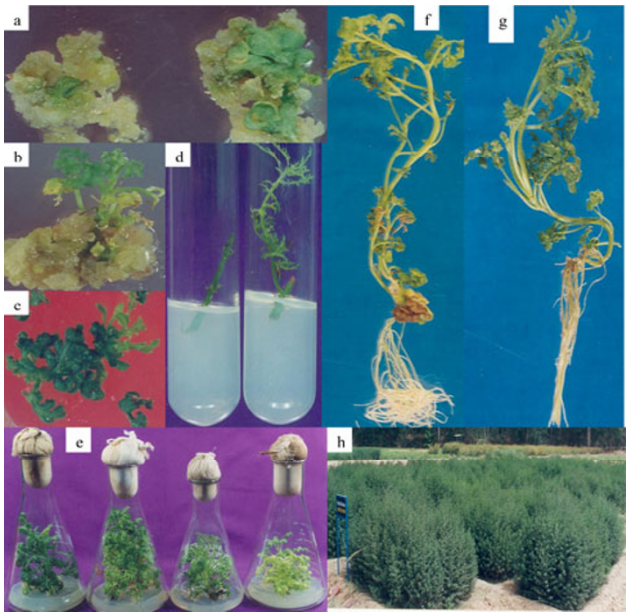

Figure 1. In vitro response of direct and indirect regeneration of *A. annua* explants
a: Callusing in leaf explants; b: Callus regeneration; c: Leaf regeneration; d: Nodal regeneration; e: Multiple shoots; f: Callus regenerated rooted plant; g: Direct regenerated rooted plant; h: Field grown mother plants

The frequency of callus formation response was found to be 63% in leaves and 35% in the nodal explants, after four weeks of culture period. This active proliferating callus, when transferred in the media containing 0.4 mg L^{-1} BAP and 0.2 mg L^{-1} NAA, exhibited shoot regeneration and served as a good source for experimentations on the indirect organogenesis through callus (Figures 1b and 1f). *In vitro* regenerated shoots obtained directly through nodal and leaf explants (Figures 1b and 1c) and indirectly through callus were maintained and multiplied on modified MS medium containing 0.2 mg L^{-1} BAP and 0.2 mg L^{-1} NAA (Table 1 and Figure 1d). The shoots on attaining a height of approximately 8–10 cm or more were then transferred on to the MSO media for rooting (Figures 1f and 1g). For hardening and establishment, these *in vitro* raised plantlets were carefully removed from the culture vessels and transferred to glass house under control conditions, like 25 ± 2 ^{o}C and 80–90% humidity, 75–80% survival rate was found after 7–10 days in glass house. After that they were transferred to the fields along with the seed-raised mother plants (Figure 1h) and tested for the genetic purity through RAPD (Random Amplified Polymoyphic DNA) profiling.

All the selected high yielding *in vitro* lines of *A. annua* was analysed for artemisinin and artemisinic acid at age of 12 weeks. Minor difference in artemisinin content in the form of decreasing order was observed in tissue culture raised plants. High yielding genotype (1.16% ± 0.041) showed 1.10% ± 0.031 artemisinin content in tissue culture. Negative correlation was observed in artemisinic acid (precursor of artemisinin) with artemisinin in both the systems (Table 3).

All the *in vitro* raised and seed raised field grown mother plants were analysed through RAPD to check the genetic fidelity. Fifty five percent polymorphism and 45% monomorphism was found in seed raised field grown mother plants whereas, in *in vitro* grown plant 42% monomorphism and 58% polymorphism were observed. These banding patterns were converted in to similarity matrix. In total 98.0% similarity was observed among the tissue culture and seed raised fields grown mother plants. As evident through RAPD profiles all the selected high yielders, which were produced through recurrent selection after four cycles[19] and simultaneously they were grown in tissue culture to maintain the genotypes, 98% similarity was observed among the field and the tissue culture raised plants. Dendrogram which was constructed using similarity matrix, showed two major clusters. Field and tissue culture control i.e. Jeewanraksha was in separate cluster from rest of the plants. The dendrogram depicts that all tissue culture raised plants were in one cluster and field raised mother plants were in another cluster except two lines with minimum difference (Figure 2).

Our preliminary studies have demonstrated the efficient protocols for maintaining the high yielding genotypes of *Artemisia annua* in tissue culture (maintaining the artemisinin content) and their comparison with seed raised field grown mother plants with respect to artemisinin content. There are few reports which have demonstrated that artemisinin can be produced successfully in callus and cell suspension cultures of *A. annua*.[20] However, some other studies have proven that artemisinin production can be enhanced by the presence of roots.[11,13] In the other report 0.287% dry weight of artemisinin can be produced in hormone-free medium when root production was maximized though no artemisinin or its immediate precursors were detected in the roots[13]. In the present study very minor difference in artemisinin content was observed in *in vitro* (12 weeks after inoculation) raised plantlets with their seed raised field grown mother plants (Tables 2 and 3). These results are an indication that differentiated shoot cultures could serve as high-value products for pharmaceutical use, since they contain artemisinin levels comparable with those observed during agricultural production. However, the low biomass produced makes it definite that tissue cultures might not be a suitable strategy for

Table 1. Effect of phytohormones supplementation in modified MS medium on the morphogenetic responses in nodal and leaf explants of *Artemisia annua* after four weeks of culture

Sr. no.	Modified MS media + BAP + NAA (mg/L)	Response (s) in %							
		Callusing		Regeneration		Proliferation		Rooting	
		Nodal	Leaf	Nodal	Leaf	Nodal	Leaf	Nodal	Leaf
1	0.1 + 0.05	35	63	-	-	-	-	-	-
2	0.2 + 0.05	-	-	84	14.5	-	-	-	-
3	0.2 + 0.2	-	-	-	-	84	-	-	-
4	0.4 + 0.2	-	-	64 (CR)	23 (CR)	-	-	-	-
5	0.5 + 0.01	-	-	-	95	-	-	-	-
6	0.5 + 0.05	-	-	-	-	23	-	-	-
7	MSO	-	-	-	-	-	-	95	95

Note: CR: Callus regeneration

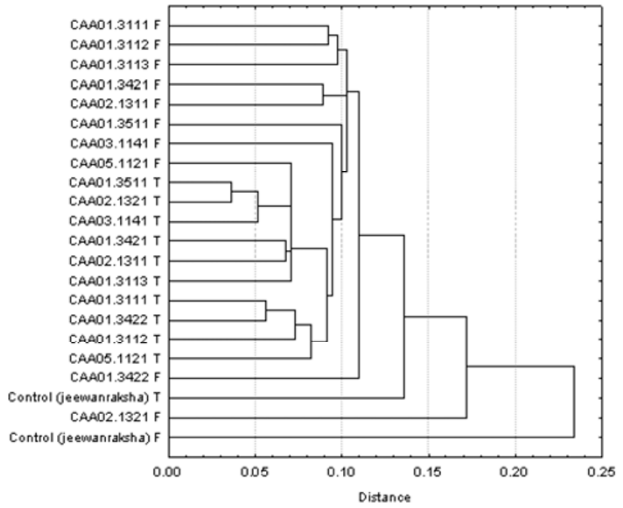

Figure 2. Dendrogram of tissue culture raised plants with field grown mother plants of *A. annua*.
Note: F is seed raised field grown mother plant and T is tissue culture raised plants

commercial exploitation of artemisinin.

Artemisinin is produced by differentiated (shoots and roots) shoot cultures[12,13] however, occurs only in shoots without roots,[9,11,14–16] these reports are congruent to our observations where the artremisinin content was observed in *in vitro* shoots only. Brown[21] reported 0.0038% of artemisinin being produced by callus cultures bearing shoots but did not specify whether or not they had roots. Jha et al[14], most authors[11,13,15,16,21,22] reported no artemisinin or only trace amounts produced from callus, cell, or the spent liquid media from these cultures. Ferreira and Janick,[13] presented evidence that artemisinin production in shoots enhanced in presence of roots[23] whereas; our study reveals the artemisinin content in the shoot only.

High level of artemisinin content found in the field condition may be attributed to the long photoperiod. At that particular time plantlets of *A. annua* were regenerated using shoot tips of mature field grown plants in tissue culture for artemisinin. Multiple shoot tips were cultured in Murashige and Skoog,[24] media with 1 mg L^{-1} BA and shoots were easily cultured using standard protocol and need cytokinin supplementation. Benzyladenine (BA) and coconut water were effective in inducing shoot from nodal explants.[12] The combination of BAP (0.2 mg L^{-1}) and NAA (0.05 mg L^{-1}) produced regeneration from nodal explants and 0.5 mg L^{-1} BAP and 0.1 mg L^{-1} NAA was used to produced direct regeneration from leaves, these combinations were used to produced true-to-type genetic material of *A. annua* (Table 1). BAP (0.2 mg L^{-1}) + NAA (0.05 mg L^{-1}) and BAP (0.2 mg L^{-1}) + NAA (0.2 mg L^{-1}) were most successful combinations in our observations which were used to generate tissue culture plantlets for further experiments.

Artemisinin was detected from *in-vitro* shoots of *A. annua*, in concentrations from 0.03 to 0.05% (dry weight basis)[25] whereas in our result after 12 weeks of culture the artemisinin content was not significantly decreased (0.9–1.0%). Maintaining artemisinin content in tissue culture may be due to specific media combinations containing 1% sugar, amine (casein hydrolysate) and extra vitamins (RT) along with BAP-NAA combination. This nutrient deficient, amine and extra vitamin may produce some stress, which may regulate terpenoid pathway (artemiainin is sesqueterpene lacton) and help to maintain artemisinin biosynthesis. Secondary metabolite synthesis may vary with the availability of nutrient[26]. Artemisinin content of *A. annua* L. plants grown under long days in a greenhouse were highly correlated with the same clones grown under long days in the field and also correlated

Table 2. Average growth increment in terms of number of branches, and leaves and the plantlet length of plants raised from the explants of eleven selected genotypes of *Artemisia annua* after 4th, 8th and 12th week of inoculation on the modified MS medium containing combinations of BAP and NAA

Sr. no.	Medium	4th week			8th week			12th week		
	Modified MS containing BAP + NAA (mg/L)	Branches (no.)	Leaf (no.)	Length of plantlet (cm)	Branches (no.)	Leaf (no.)	Length of plantlet (cm)	Branches (no.)	Leaf (no.)	Length of plantlet (cm)
1	0.2 + 0.05	1.77 ± 0.21	5.71 ± 0.30	2.3 ± 0.08	2.5 ± 0.23	11.58 ± 0.43	4.16 ± 0.15	3.25 ± 0.41	12.16 ± 0.46	4.8 ± 0.15
2	0.2 + 0.2	2.8 ± 0.26	6.5 ± 0.57	3.2 ± 0.12	2.9 ± 0.25	13.35 ± 0.34	5.29 ± 0.28	4.19 ± 0.22	14.00 ± 0.52	6.88 ± 0.19
3	0.4 + 0.2	1.51 ± 0.19	5.6 ± 0.21	2.1 ± 0.06	2.6 ± 0.22	10.22 ± 0.30	3.9 ± 0.16	3.22 ± 0.18	11.25 ± 0.41	3.5 ± 0.15
4	0.5 + 0.01	1.55 ± 0.2	5.2 ± 0.25	2.4 ± 0.08	2.5 ± 0.23	11.21 ± 0.32	4.0 ± 0.17	3.35 ± 0.24	12.15 ± 0.41	3.9 ± 0.16
5	0.5 + 0.05	1.6 ± 0.12	5.5 ± 0.29	2.2 ± 0.05	2.6 ± 0.24	11.52 ± 0.34	4.12 ± 0.11	3.28 ± 0.35	11.91 ± 0.3	4.2 ± 0.17

Note: Values represent mean ± standard error of three replicates

Table 3. Comparison of artemisinin and artemisinic acid of selected high yielding seed raised field grown and *in vitro* plants grown in the modified ½ MS media combination of 0.2 BAP + 0.2 NAA mg/L, 0.5 g L^{-1} caesine hydrolysate, 5 mg L^{-1} biotin, 2 mL L^{-1} of RT vitamin stock

Sr. no.	Lines of CIM Arogya	Artemisinin content in field grown plants		Artemisinin content in *in vitro* grown plants (after 12 weeks)	
		Artemisinin (%)	Artemisinic acid (%)	Artemisinin (%)	Artemisinic acid (%)
1	CAA01.3111	1.10 ± 0.029	0.004 ± 0.001	1.01 ± 0.015	0.004 ± 0.002
2	CAA01.3112	1.04 ± 0.015	0.009 ± 0.001	1.02 ± 0.015	0.005 ± 0.001
3	CAA01.3113	1.08 ± 0.02	0.0013 ± 0.001	0.98 ± 0.009	0.001 ± 0.001
4	CAA01.3421	1.04 ± 0.006	0.0016 ± 0.001	0.99 ± 0.032	0.001 ± 0.001
5	CAA01.3422	1.05 ± 0.01	0.001 ± 0.001	1.01 ± 0.01	0.001 ± 0.0005
6	CAA01.3511	1.12 ± 0.035	0.0067 ± 0.001	1.08 ± 0.012	0.005 ± 0.0001
7	CAA02.1311	1.04 ± 0.013	0.0012 ± 0.001	1.04 ± 0.012	0.002 ± 0.001
8	CAA02.1321	1.16 ± 0.041	0.0004 ± 0.001	1.10 ± 0.031	0.004 ± 0.001
9	CAA03.1141	1.05 ± 0.029	0.0017 ± 0.0006	0.99 ± 0.029	0.0001 ± 0.0005
10	CAA05.1121	1.05 ± 0.009	0.0052 ± 0.0006	1.01 ± 0.006	0.004 ± 0.001
11	Control (jeewanraksha)	0.90 ± 0.007	0.001 ± 0.001	0.9 ± 0.041	0.002 ± 0.001

Note: Values represent mean ± standard error of three replicates

with long days in tissue culture and in the greenhouse plants.[7]

Undoubtedly the economics of artemisinin production in plant could be drastically improved by some contemporary procedures. These are, by recurrent selection through gene pool exploitation over the generations[19] and biotechnological process.[26] It has equally been demonstrated that intermittent harvesting of the early planted full time crops within one growing season can also enhance the production of artemisinin through an increase in leaf biomass yield.[27] Taken together, these observations are highly encouraging and should be of immense significance in efforts geared towards more production of artemisinin.

Artemisinin production probably have a wider variation in plants generated from seeds[28] than in those generated from asexual propagation methods such as cuttings or *in vitro* culture, although this has not been evaluated on a large scale. Against this backdrop crop establishment from tissue culture or cloned plants looks like an attractive option if the source plant is rich in artemisinin content (more than 1%).[8,29,30] Such tissue culture methods are equally useful for maintaining genetic fidelity. However, the cost benefits of crop establishment from seeds *versus* tissue culture or asexually propagated plantlets also need to be evaluated.

Experimental Section

Plant Material, Standardization of Culture Media and Hardening. The plant materials (nodal and leaf explants) were taken from the high artemisinin yielding (more than 1%) genotypes of field growing plants of *A. annua* var. Jeewanraksha as a control[31] and eleven high yielding genotypes of var. CIM Arogya[8] at the age of 180 days of standing crop. The explants were sterilized (with 0.1% mercuric chloride for 3 min) and thoroughly washed with distilled water, were transferred in MS medium[24] supplemented with different concentration of growth hormones, 1% sucrose and solidified with 0.8% agar. The cultures were incubated at 24 °C ± 2 °C and exposed to photoperiods of 16 hrs in 300–400 lux. In order to standardised best regeneration and multiplication medium, modified half MS media supplemented with 100 mg L^{-1} myo-inositol, 0.5 g L^{-1} caesine hydrolysate, 5 mg L^{-1} biotin, 2 mL L^{-1} of RT vitamin stock (revised tobacco vitamin stock,[32] with different hormonal combinations, BAP: benzylaminopurine and NAA: naphthalene acetic acid) and 1% sucrose was used (Table 1). After a period of 60 days, plants attained substantial shoot growth and these shoots were individually transferred to MSO (MS without hormone) basal medium for rooting. Rooted plantlets were subjected to hardening and transferred to the small pots filled with soil and farm yard manure (1:1) in the glass house for 15 days maintaining 80–85% relative humidity before being transferred to the field.

For field grown mother plants, seedlings were transferred to the field in the month of February and after 180 days samples for artemisinin analysis were collected. Based on artemisinin content, explants (same age) of eleven genotypes were brought in tissue culture for multiplications and maintenance of explants with their artemisinin content.

Chemical Analysis: Artemisinin and Artemisinic acid. The antimalarial compounds, artemisinin and its precursor, artemisinic acid were estimated through HPTLC and HPLC. HPTLC technique was used to estimate the artemisinin content of *in-vitro* as well as field grown mother plants. For HPTLC, hexane extract of 0.1 g dried material was filtered and evaporated to dissolved in 1 mL hexane to spot on the pre-made TLC plate (20 × 20 cm, E-merck) at 1 cm distance along with standard (Cadila, India 1 mg mL^{-1}, 98% purity). Plate was dipped in mobile phase (1:1, diethyle ether: hexane) and pink colour spot of artemisinin was developed by spraying glacial acetic acid: H_2SO_4: anisaldehyde (50:1:0.5). The coloured spots were analyzed under densitometer (Shimadzu, Japan) for quantification.[33] Similarly, the hexane extract was dissolved in 1 mL methanol for artemisinin acid analysis, 5–10 μL of this methanolic extract was injected in HPLC (Shimatzu, Japan) using aectonitrile and water with 1% TFA (Tris fluoro-acetic acid) (70:30 v/v) as mobile phase at the flow rate of 1 mL min^{-1} with detector set at 220 nm. Pure (98%) artemisininc acid was used as standard and estimation was carried out following Gupta et al.[33]

DNA Isolation and PCR Amplification. DNA finger printing was carried out to determine the variations in selected high artemisinin yielding plants and their comparison with *in vitro* raised plants. DNA was isolated following the procedure of Khanuja et al.[34] Polymerase chain reactions (PCR) were carried out in 25 μL volume. A reaction tube contained 25 ng of DNA, 0.2 units of *Taq* DNA polymerase, 10 mM of each dNTPs, 1.5 mM $MgCl_2$ and 5 pmol of decanucleotide primers (MAP kit, 20 primers in a kit). The amplification was carried out using the DNA Engine thermal cycler (MJ Research, USA) using 94 °C, 35 °C and 72 °C temperatures for 40 cycles. The DNA profile was analyzed following Nei and Li[35] and the dendrogram of the genetic relatedness among the genotypes was produced by means of UPGMA (unweight pair group method with arithmetic average) cluster analysis.

Data Interpretation. Statistical analysis was done by following Singh and Chowdhary[36] and Statistica version 8.0.

Conclusion

In the study the elite genetic materials, which were come through unique combination of molecular genetic and breeding in *Artemisia annua* and the variation has arisen in each selected genotypes because of the highly cross-pollinated nature, was used for comparison and development of efficient protocol. To maintain the elite genotypes, in such a situation for true-to type plants, the most feasible approach is direct micro-propagation from the vegetative tissue like axillary buds, producing large number of identical multiple shoots. For this purpose, using the nodal and leaf explants of *Artemisia annua*, the conditions were optimized for media incubation towards high regeneration and rooting of the multiple shoots maintaining artemisinin content, so that the plants could be hardened and brought to the field. It also provides the information of variability at chemical and molecular level between tissue culture raised and field grown mother plants. The comparative profiles showed that artemisinin content in field grown plants was high and when it maintained in tissue culture minor difference was observed in artemisinin content after 12 week of culture. This indicates that maintaining field grown high yielder in tissue culture is good alternative and may be useful for further research and re-transplant these genotypes in field for large scale artemisinin production.

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